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(54) Title: USE OF HELIOXANTHIN AS AN ENHANCER OF CELL DIFFERENTIATION INDUCING FACTORS

(57) Abstract

An enhancer for a cell differentiation inducing factor, a composition for preventing or treating a bone disease or a nerve degenerative disease, and an osteogenesis promoter, each of which comprises helioxanthin, or its lactone-open form compound or its salt.

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USE OF HELIOXANTHIN AS AN ENHANCER OF CELL DIFFERENTIATION INDUCING FACTORS

FIELD OF THE INVENTION

The present invention relates to an enhancer for a cell differentiation inducing factor, which is useful for treating or preventing bone diseases such as osteoporosis, fractures, etc., for bone regeneration, or for treating or preventing nerve diseases such as Alzheimer's disease, cerebrovascular dementia, amyotrophic lateral sclerosis, diabetic peripheral nerve disorders (neuropathy), etc.

BACKGROUND OF THE INVENTION

The bone morphogenetic proteins (BMPs) belong to the only protein factor family isolated from decalcified bones that is known to have ectopic bone-inducing ability. Therefore it is useful as a bone - morphogenesis promoting drug for treating fractures, bone regeneration, etc. (A. E. Wang, Trends Biotechnol., Vol. 11, p. 379-383 (1993)).

Since BMP directly promotes osteoblast differentiation, BMP is considered to play a role in bone remodeling as a coupling factor and be closely related with bone metabolism. It is reported that bone matrixes in aged animals have a considerably lowered BMP content (M. L. Urist, Bone and Mineral Research, vol. 6 (ed. by W.A. Peck), p. 57-112, Elsevier, 1989), and BMP is likely to be closely related

to the maintenance of bone quantity. This suggests that BMP is a promising candidate for a remedy against various bone diseases such as osteoporosis, etc. However, BMP normally exists in only a trace amount in the living body and is available from limited sources. In addition, because BMP is a protein, there are some problems in its administration, and its subject diseases are limited.

In addition, it is reported that BMP has neurotrophic factor - like activity (V. M. Paralkar et al., J. Cell Biol., vol. 119, p. 1721-1728 (1992)). Furthermore, it is known that the BMP gene is highly expressed in brain tissues (E. Ozkaynak et al., Biochem. Biophys. Res. Commun., vol. 179, p. 116-123 (1991)). In addition, it is suggested that BMP plays an important role in neural tube formation at embryonal development (K. Basler et al., Cell, vol. 73, p. 687-702 (1993)). Therefore, BMP is considered to be closely related to neuron differentiation or maintenance of neuron function.

Neurotrophic factors belong to a group of proteinaceous factors that play important roles in the life maintenance and function expression of neurons. Neurotrophic factors include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), etc. NGF promotes the differentiation and maturation of sympathetic ganglia and dorsal root ganglia in the neural crest in the

peripheral nervous system (A. M. Davies & R. M. Lindsay, Dev. Biol., vol. 111, p. 62-72 (1985); R. Levi-Montalcini, EMBO J., vol. 6, p. 1145-1154 (1987)), and acts on cholinergic neurons of septa (the basal forebrain) in the central nervous system (H. Gnahn et al., Dev. Brain Res., vol. 9, p. 45-52 (1983); H. Hatanaka & H. Tsukui, Dev. Brain Res. vol. 30, p. 47-56 (1986); F. Hefti, J. Neurosci. vol. 6, p. 2155-2162 (1986)). NGF is essential for neuron function maintenance after completion of neuron differentiation. In the peripheral nervous system, BDNF acts on dorsal root ganglia and nodose ganglia, but not on sympathetic ganglia (R. M. Lindsay & H. Rohrer, Dev. Biol., vol. 112, p. 30-48 (1985); R. M. Lindsay et al., Dev. Biol., vol. 112, p. 319-328 (1985); A. M. Davies et al., J. Neurosci., vol. 6, p. 1897-1904 (1986)). On the other hand, in the central nervous system, BDNF acts on cholinergic neurons of septa and GABA (γ-aminobutyric acid)-ergic neurons, and dopaminergic neurons of midbrain (R. F. Alderson et al., Neuron, vol. 5, p. 297-306 (1990); C. Hyman et al., Nature, vol. 350, p. 230-232 (1991); B. Knusel et al., Proc. Natl. Acad. Sci. USA, vol. 88, p. 961-965 (1991)). Although NT-3 acts on the peripheral nervous system in a similar manner to NGF and BDNF, it is characterized by its potent action on neural placodes - derived sensory nerve cells (P. Ernfors et al., Proc. Natl. Acad. Sci. USA, vol. 87, p. 5454-5458 (1990); A. Rosenthal et al., Neuron, vol. 4, p. 767-

773 (1990)). However, any neurons in the central nervous system that respond to NT-3 have not been known.

Alzheimer's dementia shows extensive disorders and loss of cerebral cortex neurons in addition to degeneration and loss of cholinergic neurons of the basal forebrain including septa, and NGF and neurotrophic factors are considered to be candidates for remedies for the disease (F. Hefti & W. J. Weiner, Annu. Neurol., vol. 20, p. 275-281 (1986)). BDNF, a trophic factor for dopaminergic neurons of midbrain, is expected to become a remedy for Parkinson's disease, in which dopaminergic neurons of midbrain are degenerated or lost. However, because these neurotrophic factors are proteins, there is a limit for their application.

In view of the above, for example, compounds that enhance BMP activity can enhance activity of BMP present in or administered into the living body, and thus are useful as agents for treating bone diseases described above. BMP activity - enhancing substances reported so far include retinoic acid, vitamin D_3, estrogen and glucocorticoid (V. Rosen & R. S. Thies, Trends Genet., vol. 8, p. 97-102 (1992); Y. Takuwa et al., Biochem. Biophys. Res. Commun., vol. 174, p. 96-101 (1991)). However, it is known that these substances, when administered into the living body, promotes bone absorption or causes side effects such as hypercalcemia,

ovary cancer formation, etc. They are not necessarily suitable for drugs for treating osteal diseases.

On the other hand, for example, compounds that enhance NGF activity can enhance activity of NGF present in or administered into the living body, and are thus useful as drugs for treating dementia or peripheral nerve disorders. As a compound having such activity, sabeluzole (i.e., 4-(2-benzothiazolylmethyl-amino)-α [(p-fluorophenoxy)]methyl]-1-piperidineethanol) has been reported (New Current, vol. 4, No. 26, p. 14 (1993)), but its action mechanisms have not been elucidated. In addition, clinical trials have shown that sabeluzole has side effects such as headache, dizziness, fatigue, etc. Sabeluzole is thus not necessarily suitable for a drug for treating nerve diseases. As compounds having NGF secretion inducing activity, Experimental Neurology, vol. 124, p. 36-42 (1993) discloses steroids, catechols and cytokines, and USP 5059627 discloses idebenone. However, some of these compounds have adverse effects such as neurotoxicity, lowered immunity, hypercalcemia, promotion of bone absorption, etc. NGF secretion inducing activity cannot necessarily be separated from adverse effects on tissues other than the nervous system. Such compounds are thus unsatisfactory for practical use.

In addition, because cell differentiation inducing factors represented by BMP or neurotrophic factors are

proteins, there is a limit for their administration into the living body. Therefore low molecular weight compounds are preferred for compounds that enhance activity of cell differentiation inducing factors present in or administered into the living body.

Accordingly, the main object of the present invention is to find a low molecular weight compound that enhances activity of cell differentiation inducing factors represented by BMP or neurotrophic factors and to provide an enhancer for a cell differentiation inducing factor that is useful for treating or preventing various bone or nerve diseases.

This object as well as other objects and advantages of the present invention will become apparent to those skilled in the art from the following description with reference to the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the neurite outgrowth promoting ability of helioxanthin in rat pheochromocytoma cells.

Fig. 2 shows the calcification - promoting activity of helioxanthin in mouse osteoblastic cells. The calcium content is indicated as a mean ± standard deviation (SD) for 3 wells per group.

The symbol * means that there was a statistically significant difference as compared to the BMP (alone)-treatment group ($p<0.05$, t-test).

The symbol # means that there was a statistically significant difference as compared to the non-treated control group ($p<0.05$, t-test).

Fig. 3 shows the promoting action of helioxanthin on the differentiation of rat bone marrow stromal cells to osteoblasts. The alkaline phosphatase activity is indicated as a mean ± S.D. for 3 wells per group.

The symbols ** and * means that there was a statistically significant difference as compared to the non-treated control group (**: $p<0.01$, *: $p<0.05$, t-test).

Fig. 4 shows the promoting action of helioxanthin on the differentiation of rat bone marrow stromal cells to osteoblasts. The calcium content is indicated as a mean ± S.D. for 3 wells per group.

The symbols ** and * means that there was a statistically significant difference as compared to the non-treated control group (**: $p<0.01$, *: $p<0.05$, t-test).

SUMMARY OF THE INVENTION

In order to develop a drug that specifically enhances the differentiation of osteoblasts or neurons induced by BMP or neurotrophic factors, the present inventors have

intensively studied to obtain a low molecular weight compound that enhances activity of cell differentiation inducing factors. As a result, it has been found for the first time that helioxanthin or its lactone-open form compound or its salt is a potent enhancer for BMP or neurotrophic factors. Thus, the present invention has been accomplished.

The present invention provides an enhancer for a cell differentiation inducing factor, particularly for a bone morphogenetic protein or a neurotrophic factor (e.g., a nerve growth factor family), which comprises helioxanthin, or its lactone-open form compound or its salt.

The present invention also provides a composition for preventing or treating a bone disease or a nerve degenerative disease which comprises helioxanthin, or its lactone-open form compound or its salt.

The present invention also provides a composition which comprises helioxanthin, or its lactone-open form compound or its salt and a cell differentiation inducing factor.

The present invention also provides a method for enhancing a cell differentiation inducing factor which comprises using helioxanthin, or its lactone-open form compound or its salt.

The present invention also provides a method for preventing or treating a bone disease or a nerve degenerative

disease in a mammal which comprises administering to said mammal in need thereof an effective amount of helioxanthin, or its lactone-open form compound or its salt.

The present invention also provides helioxanthin, or its lactone-open form compound or its salt for use as a medicine.

The present invention also provides use of helioxanthin, or its lactone-open form compound or its salt for the manufacture of an enhancer for a cell differentiation inducing factor, or a composition for preventing or treating a bone disease or a nerve degenerative disease.

The present invention also provides a method for preparing an enhancer for a cell differentiation inducing factor or a composition for preventing or treating a bone disease or a nerve degenerative disease which comprises admixing helioxanthin, or its lactone-open form compound or its salt with a pharmaceutically acceptable carrier, excipient or diluent therefor and then subjecting the mixture to molding.

The present invention also provides a method for preparing a composition which comprises admixing helioxanthin, or its lactone-open form compound or its salt with a cell differentiation inducing factor and then subjecting the mixture to molding.

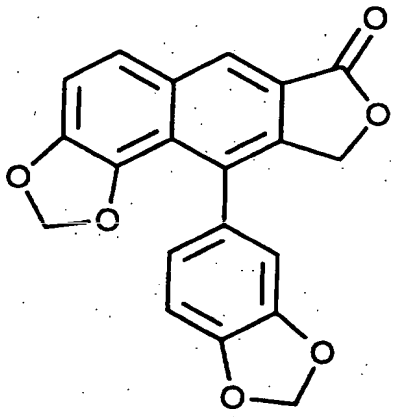
The present invention also provides an osteogenesis promoter which comprises helioxanthin, or its lactone-open form compound or its salt.

The present invention also provides use of helioxanthin, or its lactone-open form compound or its salt for the manufacture of an osteogenesis promoter.

The present invention also provides a method for preparing an osteogenesis promoter which comprises admixing helioxanthin, or its lactone-open form compound or its salt with a pharmaceutically acceptable carrier, excipient or diluent therefor and then subjecting the mixture to molding.

DETAILED DESCRIPTION OF THE INVENTION

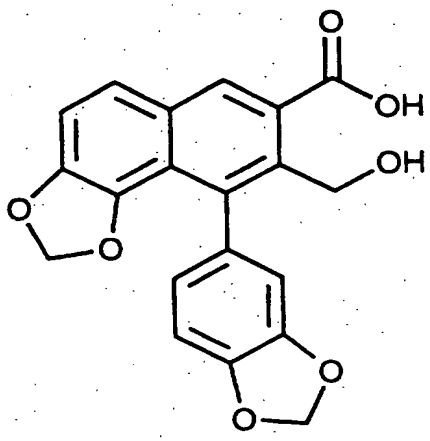
Helioxanthin (i.e., 1-(3,4-methylene-dioxyphenyl)-2-hydroxymethyl-7,8-methylenedioxy-3-naphthoic acid lactone) is a compound known as a component in polygala root (*Onji*) that is a kind of galenical derived from the plant Polygala tenuifolia Willd (R. S. Burden et al., J. Chem. Soc. (C), 693-701 (1969)). The structure of helioxanthin is represented by the formula (I) below, and is quite different from that of substances having activity of enhancing BMP or NGF activity.



(I)

Helioxanthin can be chemically synthesized as described in J. of Natural product, vol. 52(2), p. 367-375 (1989).

The lactone-open form compound of helioxanthin, having the formula (II):



(II)

can be obtained by hydrolyzing the lactone moiety in helioxanthin by a per se known method to form a carboxyl group and a hydroxyl group.

The salt of the lactone-open form compound (II) is preferably a physiologically acceptable salt. Examples of the salts include salts with inorganic bases, organic bases, inorganic acids, organic acids, basic or acidic amino acids, etc. Preferred examples of the salts with inorganic bases include salts with alkaline metals such as sodium, potassium, etc.; salts with alkaline earth metals such as calcium, magnesium, etc.; salts with aluminium; etc. Preferred examples of the salts with organic bases include ammonium salts, salts with trimethylamine, triethylamine, pyridine, picoline, ethanolamine, diethanolamine, triethanolamine, dicyclohexylamine, N,N'-dibenzylethylenediamine, etc. Preferred examples of the salts with inorganic acids include salts with hydrochloric acid, hydrobromic acid, nitric acid, sulfuric acid, phosphoric acid, etc. Preferred examples of the salts with organic acids include salts with formic acid, acetic acid, trifluoroacetic acid, fumaric acid, oxalic acid, tartaric acid, maleic acid, citric acid, succinic acid, malic acid, methanesulfonic acid, benzenesulfonic acid, p-toluenesulfonic acid, etc. Preferred examples of the salts with basic amino acids include salts with arginine, lysine, ornithine, etc. Preferred examples of the salts with acidic amino acids include salts with aspartic acid, glutamic acid, etc. These salts can be obtained by conventional methods.

The cell differentiation inducing factors in the present invention include bone morphogenetic proteins, neurotrophic factors, factors belonging to transforming growth factor (TGF) - β superfamily such as TGF-β, activin, etc., factors belonging to fibroblast growth factor (FGF) superfamily such as basic fibroblast growth factor (bFGF), acidic fibroblast growth factor (aFGF), etc., factors belonging to neuropoietic cytokine family such as leukemia inhibitory factor (LIF) (also referred to as cholinergic differentiation factor (CDF)), cilialy neurotrophic factor (CNTF), etc., factors that induce characters characteristic of the step where cells maintaining living function differentiate from indifferent precursors in specific tissues such as osteoblasts or neurons, such as interleukin-1 (IL-1, hereinafter abbreviated likewise), IL-2, IL-3, IL-5, IL-6, IL-7, IL-9, IL-11, tumor necrosis factor - α (TNF-α), interferon - γ (INF-γ). In particular, bone morphogenetic proteins and neutrophic factors are preferred.

The bone morphogenetic proteins include proteins that promote bone and cartilage formation, such as BMP family (e.g., BMP-2, -4, -5, -6, -7, -8, -9, -10, -11, -12, etc), in particular, BMP-2, -4, -6, and -7. BMP may be in the form of homodimers of each factor described above or heterodimers of possible combinations of the above factors.

The neurotrophic factors include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), glia derived neurotrophic factor (GDNF), NT-4/5, etc. Preferred examples thereof are factors belonging to a nerve growth factor family, such as NGF, BDNF, and NT-3.

The enhancer of the invention (including the compositions of the invention) can be used alone or in combination with substances having cell differentiation inducing activity (e.g., BMP, neurotrophic factors) to promote fracture healing and bone regeneration and treat or prevent various bone diseases such as osteoporosis, etc., nerve degenerative disorders in cerebrovascular dementia, senile dementia, Alzheimer's disease, etc., various cerebral function disorders or nerve diseases such as amyotrophic lateral sclerosis, diabetic peripheral nerve disorders (neuropathy), etc. The enhancer of the invention can also be used as a therapeutic or prophylactic drug against diseases associated with BMP, neurotrophic factors, etc.

The enhancer of the invention can be applied to the above diseases in humans and other mammals (e.g., mice, rats, rabbits, dogs, cats, cattle, swine, etc.).

The enhancer of the invention can be administered orally or parenterally to humans.

The enhancer of the invention can be prepared by known methods in the art except that helioxanthin or its lactone-open form compound or its salt is formulated.

In the present invention, helioxanthin or its lactone-open form compound or its salt can be used alone or in combination with a physiologically acceptable carrier. When the physiologically acceptable carrier is combined, the amount of helioxanthin or its lactone-open form compound or its salt can be appropriately selected depending on the kind of preparation. However, the amount of helioxanthin or its lactone-open form compound or its salt contained in the composition of the invention is normally about 0.3 to 100% by weight, preferably about 0.5 to 20% by weight.

Compositions for oral administration include solid or liquid dosage forms such as tablets (including sugar-coated tablets and film-coated tablets), granules, powders, capsules (including soft capsules), syrups, emulsions, suspensions, etc. These compositions can be prepared by per se known methods and contain carriers commonly used in the art. Such carriers include physiologically acceptable carriers that are organic or inorganic materials commonly used for pharmaceutical carriers. The carriers include excipients, lubricants, binders, disintegrators, etc. for solid preparations; and suspending agents for liquid preparations. If necessary, appropriate additives such as antiseptics,

antioxidants, colorants, sweetening agents, etc. can be used in appropriate amounts.

Preferred examples of the excipients include lactose, sucrose, D-mannitol, starch, crystalline cellulose, light anhydrous silicic acid, etc.

Preferred examples of the lubricants include magnesium stearate, calcium stearate, talc, colloidal silica, etc.

Preferred examples of the binders include crystalline cellulose, sucrose, D-mannitol, dextrin, hydroxypropylcellulose, hydroxypropylmethylcellulose, polyvinylpyrrolidone, etc.

Preferred examples of the disintegrators include starch, carboxymethylcellulose, carboxymethylcellulose calcium, croscarmellose sodium, carboxymethyl starch sodium, etc.

Preferred examples of the suspending agents include surfactants such as stearyl triethanolamine, sodium lauryl sulfate, laurylaminopropionic acid, lecithin, benzalkonium chloride, benzethonium chloride, glyceryl monostearate, etc.; hydrophilic polymers such as polyvinyl alcohol, polyvinyl pyrrolidone, carboxymethyl cellulose sodium, methylcellulose, hydroxymethylcellulose, hydroxyethylcellulose, hydroxypropyl-cellulose , etc.

Preferred examples of the antiseptics include parahydroxybenzoic acid esters, chlorobutanol, benzyl alcohol, phenethyl alcohol, dehydroacetic acid, sorbic acid, etc.

Preferred examples of the antioxidants include sulfites, ascorbic acid, etc.

Compositions for parenteral administration include, for example, injections, suppositories, etc. The injections include subcutaneous, intradermal (or intracutaneous), or intramuscular injections. Such injections can be prepared as aqueous solutions by per se known methods, for example, by dissolving, suspending or emulsifying helioxanthin or its lactone-open form compound or its salt in sterile aqueous or oily liquids commonly used for injections. The aqueous liquids for injections include physiological saline, isotonic solutions, etc. which can optionally be used in combination with appropriate suspending agents such as carboxymethylcellulose sodium, nonionic surfactants, etc. in appropriate amounts. The oily liquids include sesame oil, soybean oil, etc. which can optionally be used in combination with solution adjuvants such as benzyl benzoate, benzyl alcohol, etc. Normally, the liquid for injection thus prepared is filled into an appropriate ampule.

The dose for a particular patient can be determined depending on the age, body weight, physical conditions, sex, diets, administration period, administration methods,

clearance, combinations of drugs, severity of the disease to be treated, or other factors.

The dose of the enhancer of the invention can appropriately be selected depending on the kind or severity of disease, etc. For example, in the case of oral administration to an adult patient (body weight: 50 kg) with osteoporosis, the enhancer containing about 0.1 to 500 mg, preferably about 1 to 50 mg, more preferably about 3 to 50 mg, of helioxanthin or its lactone open form compound or its salt is administered per day. The unit dose can be determined, considering such a daily dose, dosage forms, etc. The frequency of administrations is not specifically limited, and is preferably 1 to 5 times a day, more preferably 1 to 3 times a day.

As described above, helioxanthin is known as a component of a galenical and has low toxicity.

Since the enhancer of the invention has potent bone formation - promoting activity, it can be mixed with a carrier for bone regeneration to prepare a bone formation - promoting drug for bone repair or bone implantation. For example, the enhancer of the invention may be attached or added to artificial bones, etc., made from metals, ceramics or polymers. The artificial bones preferably have many pores on the surface so as to release the enhancer of the invention in living tissues when they are implanted in a bone - defective

part. A dispersion of helioxanthin in an appropriate disperser, binder, diluent, etc. (e.g., collagen, physiological saline, citric acid solution, acetic acid solution, hydroxyapatite, fibrin, mixed solutions thereof, etc.) may be applied to or impregnated into artificial bones, followed by drying the bones. In this manner, the enhancer of the invention can be attached to or incorporated into artificial bones. Such artificial bones are implanted in a bone - defective part and tightly fixed to the bone - defective part. The fixing agent for artificial bones can be prepared by mixing the active ingredient helioxanthin with a physiologically acceptable dispersion, binder, diluent, other ingredients effective for bone regeneration (e.g., calcium), etc. The fixing agent for artificial bones can also be used so as to fill the gaps between the artificial bones to be implanted in the bone - defective part and the bone - defective part in the host without attaching or incorporating it into artificial bones. In addition, bone morphogenesis - promoting proteins such as BMP family may be attached to or contained in the above parenteral compositions.

EXAMPLES

The following experiments and examples further illustrate the present invention in detail but are not to be construed to limit the scope thereof.

Experiment 1

Induction of alkaline phosphatase (ALP) production in a mouse osteoblast strain

The mouse-derived osteoblast strain MC3T3-E1 was inoculated (8000/well) in a 96-well plate in an α-minimum essential medium (MEM) containing 10% fetal calf serum (FCS). Two days later, the sample diluted to the concentrations described in Table 1 with a medium containing or not containing BMP-4/7 heterodimer (described in Japanese Patent Application No. 6-111255) (3 ng/ml) was added to cells that had been confluent all over the surface, and the mixture was incubated for 72 hours. After the plate was washed with physiological saline once, the substrate solution was added, and the mixture was incubated at room temperature for 15 minutes. The reaction was stopped by adding 0.05N sodium hydroxide, and the absorbance at 405 nm was measured. The results are shown in Table 1. The results show that helioxanthin enhances BMP activity, i.e., induction of ALP production by BMP, and that helioxanthin alone has potent ALP production - inducing activity regardless of the presence or absence of BMP.

Table 1

Induction of ALP production in mouse osteoblastic cells (MC3T3-E1)

Final concentration of helioxanthin (M)	ALP activity ($1000 \times A_{405} \pm SD$) In the presence of BMP (3 ng/ml)	In the absence of BMP
0 (control: no addition)	94± 7	35± 3
1.6×10^{-8}	162±12 *	59± 4 *
1.3×10^{-7}	336±39 *	106±11 *
1.0×10^{-6}	574±55 *	239±11 *

* : Statistically significant ($p<0.001$ vs control; t-test)

Experiment 2

Neurite outgrowth in rat pheochromocytoma

PC 12 cells (rat pheochromocytoma; 2000/well) suspended in Dulbecco MEM containing 10% FCS were mixed with the sample containing varying concentrations of NGF and helioxanthin shown in Fig. 1, inoculated in a 96-well plate, and then cultivated for 3 days. The culture solution was removed, and hematoxylin-eosin staining was conducted using a commercially available kit (Diff-Quik R, International Reagents Corporation, Kobe, Japan). The cells were observed with a microscope to evaluate the neurite outgrowth. The results are shown in Fig. 1. Significant outgrowth of the neurites of the sample cells were observed when helioxanthin

(1 μM or 10 μM) was added in the presence of NGF (10 ng/ml). That is, the addition of helioxanthin (1 μM or 10 μM) in the presence of NGF (10 ng/ml) produced equivalent or higher effect than the treatment of NGF (100 ng/ml) alone. Thus, the results show helioxanthin enhances the action of nerve growth factors.

Experiment 3

Calcification - promoting activity of helioxanthin in a mouse osteoblastic cells

The mouse osteoblastic cell line, MC3T3-E1, was inoculated (10^4/well) in a 24-well plate. From the next day, the cells were cultivated in α-MEM medium that contains 10% FCS containing various concentrations of helioxanthin, 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid in the presence or absence of 3 ng/ml of BMP for 10 days. The cells were rinsed once with phosphate-buffered saline (PBS), and then 6N hydrochloric acid (0.2 ml) was added. After 5 minutes, this solution was recovered to determine the calcium content with a colorimetry kit (Calcium E - Test Wako, Wako Pure Chemical Industries, Ltd.) (n=3). The results are shown in Fig. 2. The results show that treatment with BMP alone did not cause clear calcification, whereas treatment with BMP in combination with 10 μM or 1 μM helioxanthin significantly promoted calcification. That is, the results show that helioxanthin significantly enhances differentiation inducing action of BMP.

Treatment with 10 μM helioxanthin alone significantly promoted calcification as compared to the non-treatment control group. This is probably because helioxanthin enhanced endogenous BMP-like action.

Experiment 4

Promoting action of helioxanthin on the differentiation of rat bone marrow stromal cells to osteoblasts

Stromal cells obtained from femora of 5-week-old SD rats were inoculated (10^4/well) in a 24-well plate. From the next day, the cells were cultivated in α-MEM medium that contains 15% FCS containing various concentrations of helioxanthin, 10 mM β-glycerophosphate, 50 μg/ml ascorbic acid and 10^{-7}M dexamethasone for 13 days. On the 4th, 7th, 11th and 13th days from the beginning of the cultivation, the cells were rinsed once with PBS, and a substrate solution (see Experiment 1) was directly added to the cells to determine alkaline phosphatase activity (n=3). In addition, 6N hydrochloric acid (0.2 ml) was added, and the solution was recovered to determine the calcium content with a colorimetry kit (Calcium E - Test Wako, Wako Chemical Industries, Ltd.) (n=3). The results are shown in Fig. 3. As shown in Fig. 3, helioxanthin increased alkaline phosphatase activity in a dose-dependent manner. The increase in alkaline phosphatase activity became significant as the cultivation term became

longer. As shown in Fig. 4, in the helioxanthin-treatment groups, calcification was suddenly promoted from the 11th day from the beginning of the cultivation. The results show that helioxanthin acts on precursor cells of osteoblasts in the living body to promote its maturation and differentiation. This action of helioxanthin is considered to result from the enhancement of stimulation of a low concentration of endogenous BMP.

Example 1

The following ingredients (1) to (6) are mixed. From the mixture, 1000 raw tablets each containing helioxanthin 5 mg and being 6.5 mm in diameter are obtained. The raw tablets are coated with the ingredients (7) to (9) to obtain film-coated tablets of 6.6 cm in diameter.

(1) Helioxanthin	5.0 g
(2) Lactose	82.5 g
(3) Hydroxypropylcellulose	2.8 g
(4) Magnesium stearate	0.4 g
(5) Hydroxypropylmethylcellulose 2910	2.994 g
(6) Corn starch	19.3 g
(7) Macrogol 6000	0.6 g
(8) Titanium oxide	0.4 g
(9) Iron sesquioxide	0.006 g

Example 2

The following ingredients (1), (3), (4), (5), (6), (7) and (8) are suspended or dissolved in purified water, and the nucleus granules of the ingredient (2) are coated with the suspension or solution to obtain raw fine granules. The raw fine granules are coated with the ingredients (9) to (11) to obtain coated fine granules. They are mixed with the ingredient (12) to obtain helioxanthin fine granules (1%, about 500 g). The fine granules are wrapped so that each wrapper contains about 500 mg of the fine granules.

(1) Helioxanthin	5 g
(2) Lactose-crystalline cellulose (granules)	330 g
(3) D-mannitol	29 g
(4) Low substituted hydroxypropylcellulose	20 g
(5) Talc	25 g
(6) Hydroxypropylcellulose	50 g
(7) Aspartame	3 g
(8) Dipotassium glycyrrhizinate	3 g
(9) Hydroxypropylmethylcellulose 2910	30 g
(10) Titanium oxide	3.5 g
(11) Yellow iron sesquioxide	0.5 g
(12) Light anhydrous silicic acid	1 g

As described above, the enhancer of the invention has, for example, potent BMP action - enhancing activity and

bone morphogenesis - promoting activity, and acts on bone tissues to increase bone weight and strength. The enhancer is, therefore, useful for treating or preventing various bone diseases such as osteoporosis, or promoting fracture healing or bone regeneration, etc. In addition, the enhancer of the invention enhances the activity of neutrophilic factors, and is useful for treating or preventing various nerve diseases such as Alzheimer's dementia, senile dementia, mononeuron disorders (e.g., amyotrophic lateral sclerosis, etc.), diabetic peripheral nerve disorders, etc.

CLAIMS

1. An enhancer for a cell differentiation inducing factor, which comprises helioxanthin, or its lactone-open form compound or its salt.

2. An enhancer according to claim 1, wherein the cell differentiation inducing factor is a bone morphogenetic protein.

3. An enhancer according to claim 1, wherein the cell differentiation inducing factor is a neurotrophic factor.

4. An enhancer according to claim 3, wherein the neurotrophic factor is a nerve growth factor family.

5. A composition for preventing or treating a bone disease which comprises helioxanthin, or its lactone-open form compound or its salt.

6. A composition for preventing or treating a nerve degenerative disease which comprises helioxanthin, or its lactone-open form compound or its salt.

7. A composition which comprises helioxanthin, or its lactone-open form compound or its salt and a cell differentiation inducing factor.

8. A method for enhancing a cell differentiation inducing factor which comprises using helioxanthin, or its lactone-open form compound or its salt.

9. A method for preventing or treating a bone disease in a mammal which comprises administering to said mammal in need thereof an effective amount of helioxanthin, or its lactone-open form compound or its salt.

10. A method for preventing or treating a nerve degenerative disease in a mammal which comprises administering to said mammal in need thereof an effective amount of helioxanthin, or its lactone-open form compound or its salt.

11. Helioxanthin, or its lactone-open form compound or its salt for use as a medicine.

12. Use of helioxanthin, or its lactone-open form compound or its salt for the manufacture of an enhancer for a cell differentiation inducing factor.

13. Use of helioxanthin, or its lactone-open form compound or its salt for the manufacture of a composition for preventing or treating a bone disease.

14. Use of helioxanthin, or its lactone-open form compound or its salt for the manufacture of a composition for preventing or treating a nerve degenerative disease.

15. A method for preparing an enhancer for a cell differentiation inducing factor which comprises admixing helioxanthin, or its lactone-open form compound or its salt with a pharmaceutically acceptable carrier, excipient or diluent therefor and then subjecting the mixture to molding.

16. A method for preparing a composition for preventing or treating a bone disease which comprises admixing helioxanthin, or its lactone-open form compound or its salt with a pharmaceutically acceptable carrier, excipient or diluent therefor and then subjecting the mixture to molding.

17. A method for preparing a composition for preventing or treating a nerve degenerative disease which comprises admixing helioxanthin, or its lactone-open form compound or its salt with a pharmaceutically acceptable carrier, excipient or diluent therefor and then subjecting the mixture to molding.

18. A method for preparing a composition which comprises admixing helioxanthin, or its lactone-open form compound or its salt with a cell differentiation inducing factor and then subjecting the mixture to molding.

19. An osteogenesis promoter which comprises helioxanthin, or its lactone-open form compound or its salt.

20. Use of helioxanthin, or its lactone-open form compound or its salt for the manufacture of an osteogenesis promoter.

21. A method for preparing an osteogenesis promoter which comprises admixing helioxanthin, or its lactone-open form compound or its salt with a pharmaceutically acceptable carrier, excipient or diluent therefor and then subjecting the mixture to molding.

Fig. 1

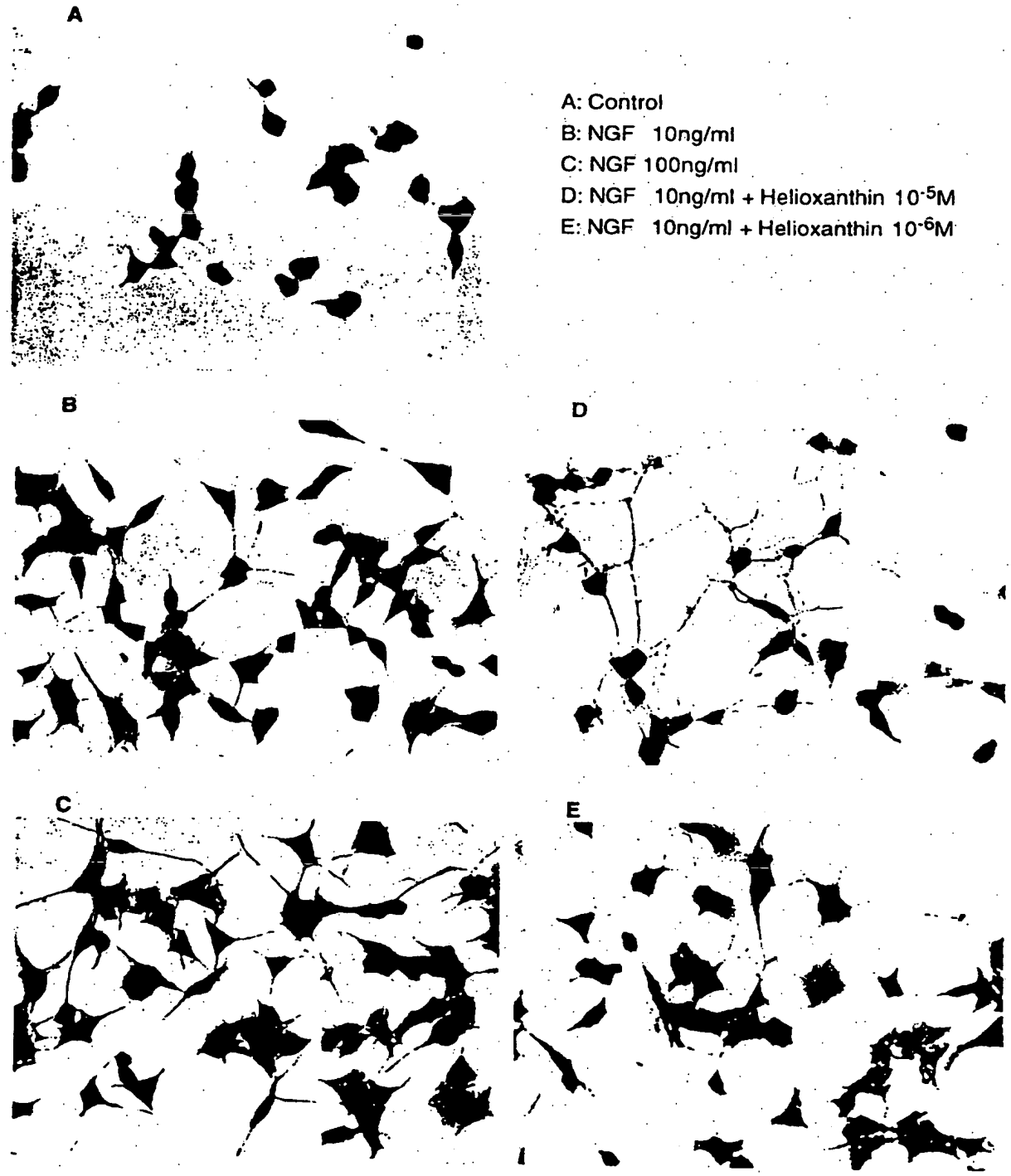

A
B
C
D
E
A: Control
B: NGF 10ng/ml
C: NGF 100ng/ml
D: NGF 10ng/ml + Helioxanthin 10^{-5}M
E: NGF 10ng/ml + Helioxanthin 10^{-6}M

Fig. 2

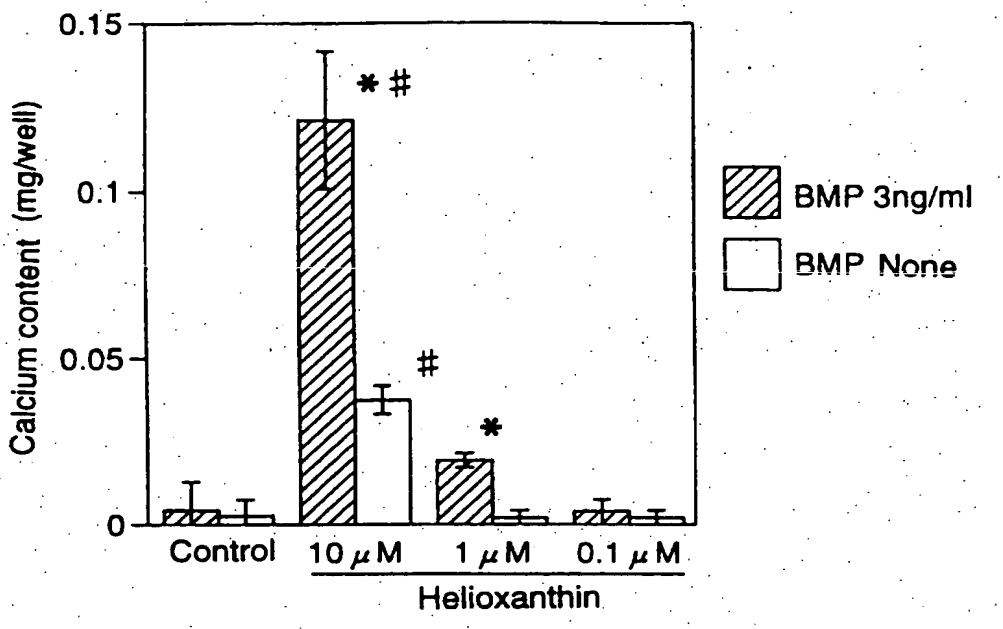

0.15
0.1
0.05
0
Calcium content (mg/well)
* #
#
*
Control
10 μM
1 μM
0.1 μM
Helioxanthin
BMP 3ng/ml
BMP None

Fig. 3

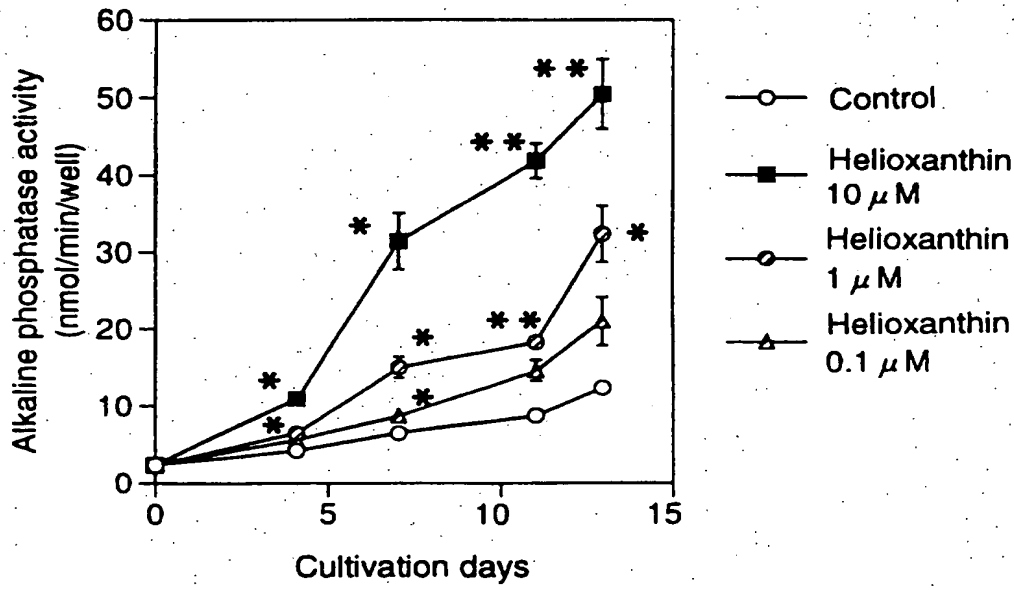
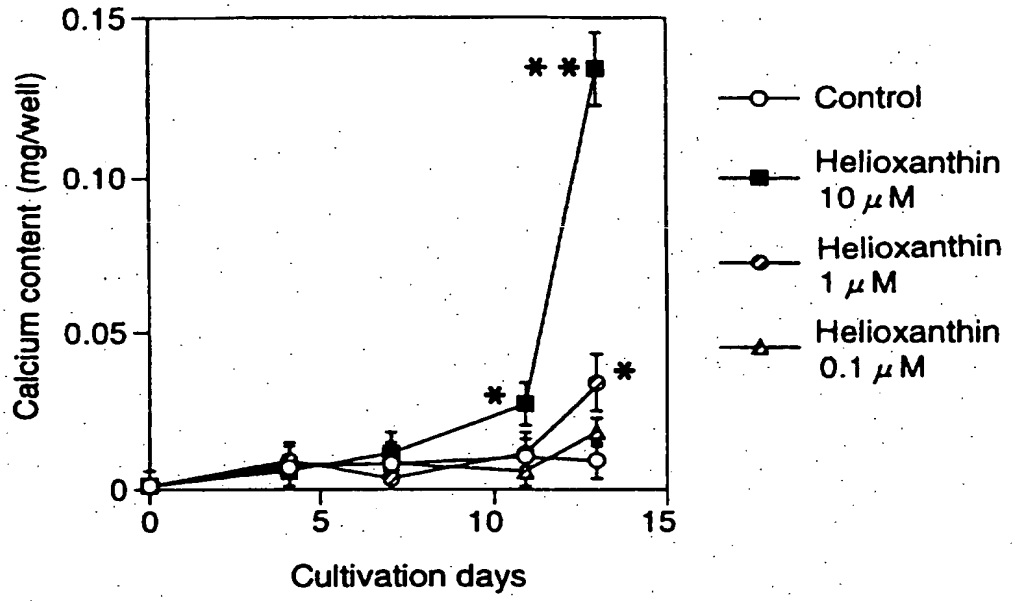

60
50
40
30
20
10
0
Alkaline phosphatase activity
(nmol/min/well)
0
5
10
15
Cultivation days
Control
Helioxanthin
10 μM
Helioxanthin
1 μM
Helioxanthin
0.1 μM

Fig. 4


0.15
0.10
0.05
0
Calcium content (mg/well)
0
5
10
15
Cultivation days
Control
Helioxanthin 10 μM
Helioxanthin 1 μM
Helioxanthin 0.1 μM

INTERNATIONAL SEARCH REPORT

International Application No
PCT/JP 96/00375

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/36 A61K31/365

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE WPI Section Ch, Week 9238 Derwent Publications Ltd., London, GB; Class B02, AN 92-310728 XP002009377 & JP,A,04 211 609 (TAKEDA CHEM IND LTD) , 3 August 1992 see abstract ---	1-21
A	DATABASE WPI Section Ch, Week 9105 Derwent Publications Ltd., London, GB; Class A96, AN 91-031960 XP002009378 & JP,A,02 300 124 (TAKEDA CHEMICAL IND KK) , 12 December 1990 see abstract --- -/--	1-21

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 July 1996	01.08.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Mair, J

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE WPI Section Ch, Week 9509 Derwent Publications Ltd., London, GB; Class B02, AN 95-063775 XP002009379 & JP,A,06 340 528 (SUNTORY LTD) , 13 December 1994 see abstract	1-21
A	EXPERIMENTAL NEUROLOGY, vol. 124, no. 1, 1993, pages 36-42, XP000576599 CARSWELL, S.: "The potential for treating neurodegenerative disorders with NGF-inducing compounds" cited in the application especially page 38, table 2A see the whole document	1-21
A	TRENDS IN BIOTECHNOLOGY, vol. 11, 1993, pages 379-383, XP000576034 WANG, E.A.: "Bone morphogenetic proteins (BMPs): therapeutic potential in healing bony defects" cited in the application see the whole document	1-21
X	TETRAHEDRON LETTERS, vol. 9, 1968, pages 2091-2094, XP000576112 BURDEN, R.S. ET AL: "Extractives of Heliopsis scabra: structure" see the whole document especially page 1035, compound I & page 1036, compound VI	1-4,11, 19
X	JOURNAL OF NATURAL PRODUCTS, vol. 43, no. 4, 1980, pages 482-486, XP000576809 OLANIYI, A.A.: "Lignans from Justicia flava" see the whole document epecially page 484, line 40-44	1-7,11, 15-19
A	WO,A,94 27614 (CONPHARM AB ;ANALYTECON SA (CH); ROSEN BOERJE (SE); LEANDER KURT () 8 December 1994 especially claims 8 & 9 see the whole document	1-21

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int[redacted]al Application No
PCT/JP 96/00375

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9427614	08-12-94	AU-B- 6939894	20-12-94
		BR-A- 9406655	19-03-96
		CA-A- 2163887	08-12-94
		CZ-A- 9503106	17-04-96
		EP-A- 0700296	13-03-96
		NO-A- 954806	26-01-96